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T Cell Activation

Field of the Invention

5 The present invention relates to an antigen independent
method for the activation of T cells. The invention also
relates to a method for increasing lymphokine production in
a T cell culture and a method for increasing the immune
response at specific sites *in vivo* which has therapeutic
10 applications in the treatment of disease.

Background to the Invention

T cells are involved in the immune response and are
15 primarily involved in cellular immunity, such as guarding
against virally infected cells, fungi, parasites and foreign
tissue.

Briefly, T cells are activated by binding to antigen-
20 displaying macrophages. However, the T cell receptor must
specifically complex with the antigen and a Major
Histocompatibility Complex (MHC) protein displayed on the
surface of the macrophage.

25 The binding induces the macrophage to release interleukin-
1, a polypeptide growth factor, which stimulates the bound
T cell to proliferate and differentiate. This proliferation
and differentiation is enhanced by the T cells
autostimulatory secretion interleukin-2. The T cell can
30 differentiate into a number of different phenotypes, such as
cytotoxic T cells which are specifically targeted to antigen
displaying host cells and are capable of lysing the cell,
helper T cells which are involved in activating cytotoxic T
cells and in co-operating with B cells to produce antibodies
35 and memory T cells which upon re-encountering their cognate
antigen proliferate at a faster rate than non-memory T
cells.

It will be apparent to one skilled in the art that the activation of T cells is an important step in the immunological response. By manipulating the activation of T cells it will be possible to obtain useful immunological 5 products and develop more efficient treatment techniques.

Previously, to achieve T cell activation, a macrophage displaying an antigen and an MHC protein was required. A number of problems and drawbacks are associated with this, 10 a major drawback being that only T cells specific for the antigen are activated. Other T cells not specific for the antigen remain unactivated. Other problems may arise if the desired antigen is difficult to obtain or hazardous to work with. Additionally, if an antigen is used in cell cultur‑ 15 to achieve activation and it is not easy to remove, contamination problems may occur.

The same problems will occur *in vivo* and it is obviously undesirable to infect an individual with an antigenic 20 substance.

By achieving antigen independent T cell activation it wil¹ be possible to activate a population of T cells without the need to isolate and display an antigen on the surface of ⸱ 25 macrophage.

It is known that interleukin-2 is potent T-lymphocyte growt‑ enhancer and the use of interleukin-2 as an adjuvant h⸱ been described. In this role interleukin-2 was thought ⁺ 30 function as an expander of the population of alrea⸱ activated T-lymphocytes. However, it was not known tha⁺ interleukin-2 (in combination with other cytokines) coul act specifically to activate T-lymphocytes in an antige⸱ independent manner.

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Summary of the Invention

According to the present invention there is provided a

method for antigen independent activation of T cells comprising contacting T cells with a combination of cytokines.

5 Preferably, the T cells are contacted with at least two of the following:

     i)   interleukin-2;

     ii) interleukin-6; and

10   iii) tumour necrosis factor α

or functionally equivalent fragments thereof.

The T cells may be naive T cells and/or memory resting T
15 cells, most suitably naive CD45RA* cells and/or memory resting CD45RO* cells.

Suitably, the concentration of interleukin-2 is from 100 to 400 U/ml, the concentration of interleukin-6 is from 400 to
20 600 U/ml and the concentration of tumour necrosis factor α is from 15 to 35 ng/ml. More preferably, the concentration of interleukin-2 is from 200 to 300 U/ml, the concentration of interleukin-6 is about 500 U/ml and the concentration of tumour necrosis factor α is about 25 ng/ml.

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The T cells may be activated in vitro, for example, in a method for obtaining increased lymphokine production from a T cell culture, comprising activating the T cells according to the invention.

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The T cells wherein T cells may be activated in vivo, leading to an enhanced immunological response which may be used in a method of therapy comprising activating in a human or animal subject T cells using the method according to the
35 inventi n.

In this aspect of the invention, the combination of cytokines acts as an adjuvant enhancing the T-cell response

4

and thereby enhancing the immune response.

T cells can be activated to produce desirable lymphokines
useful in cell-mediated immune responses, such as
5 interleukins, interferons and colony stimulating factors,
without the problems associated with antigen dependent
activation.

Additionally, it will be possible to achieve isolated T cell
10 activation and effector T cell recruitment in areas of
specific immunological interest without the use of antigens.
This will thus be extremely useful for the *in vivo* treatment
of numerous diseases and infections such as HIV and
Hepatitis.
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The present invention has the advantages of activating "by-
stander" T cells, not just specifically one particular
stimulating antigen, thus a bigger immune response is
produced leading to the production of more lymphokines and
20 subsequently greater immunoglobulin production by B cells.

Another advantage of the present invention is the
maintenance of the peripheral pool of memory T cells as
memory T cells can be expanded (proliferated) without the
25 need of specific antigenic stimulation to maintain the
clonal size. Also the naive T cell repertoire can be
maintained, as the present invention allows the
proliferation of naive T cells without them switching to the
memory phenotype, unlike in antigenic stimulation.
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According to a further aspect of the invention there is
provided a pharmaceutical composition comprising two or more
of the following:

35   i)   interleukin-2;
       ii) interleukin-6; and
       iii) tumour necrosis factor α

or functionally quivalent fragments there f opti nally in association with one or more pharmaceutically acceptable excipients.

5   The pharmaceutical composition may itself be useful for the therapeutic activation of T-cells or may be administered with a further therapeutic agent such as a vaccine. Administration may be simultaneous or sequential.

10 According to the present invention there is provided a method of gene therapy comprising the step of administering a vector carrying a genes encoding two or more of

        i)   interleukin-2;
15   ii) interleukin-6; and
        iii) tumour necrosis factor α

or functionally equivalent fragments thereof.

20 Suitable such vectors are well known in the art[1].

According to a further aspect of the invention, there is provided a combined method of therapy comprising coadministration of a vector carrying a gene encoding one 25 or more of

        i)   interleukin-2;
        ii) interleukin-6; and
        iii) tumour necrosis factor α

30
or functionally equivalent fragments thereof

and one or more of

35   i)   interleukin-2;
        ii) interleukin-6; and

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[1]Reference?

iii) tumour necrosis factor α

proteins or functionally equivalent fragments thereof.

5   Such maintenance of specific T cell types is extremely advantageous when working with T cell cultures.

Many other uses and advantages can be seen for the present invention and such uses and advantages would be apparent to
10   one skilled in the art.

Brief Description of the Drawings

Figure 1.   Phenotypic and cell cycle analysis of purified
15   CD4⁺ resting T cells.   (A) forward and side scatter profile.
(B) Cell cycle analysis.   (C) FITC- or PE-conjugated control antibodies.   (D-F) Purity of CD4⁺ cells and expression of activation markers.   (G) Expression of CD45RA and CD45RO Ags on sorted CD4⁺ cells.   (H and I) CD4⁺ cells purified as
20   CD45RO⁺ or CD45RA⁺ subpopulations.

Figure 2.   Activation of resting CD4⁺ T cells by soluble factors.   (A and B) Expression of activation markers on resting T cells cultured with supernatant from T cell clones
25   cultured with autologous macrophages prepulsed with Ag (hatched bars) or medium (solid bars), or rIL-2 (open bars). Expression of CD69 or CD25 was analyzed in double staining with anti-CD4.   (C) [³H]Thymidine incorporation of the same cells in A and B, cultured with medium alone (triangles),
30   rIL-2 (squares), or supernatant from a T cell clone cultured with macrophages prepulsed with Ag (closed circle) or medium (open circle).   (D) [³H]Thymidine incorporation of resting CD45RO⁺ (squares) or CD45RA⁺ (circles) T cells in the presence of different concentration of IL-2 plus 1 μg/ml LPS
35   (open symbols), or IL-2 with supernatant from LPS-activated macr phag s (closed symbols).

Figure 3.   C mbination of IL-2, TNF-α, and IL-6 activates

7

resting T cells. CD45RO* (*A*) or CD45RA* (*B*) resting T cells
were cultured for 8 d with various combinations of the
following: rIL-2, rIL-6, TNF-α, and supernatant from LPS-
stimulated macrophages. Thymidine incorporation and CD69
5   expression were measured as described in Fig. 1. (*C*) Cell
cycle analysis of resting CD45RO* (*squares*) or CD45RA*
(*circles*) T cells in the presence of IL-2 alone (*open
symbols*) or in combination with TNF-α and IL-6 (*closed
symbols*).

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Figure 4. CD45RA* T cells activated by cytokines do not
switch their phenotype to CD45RO. CD45RA* T cells were
activated by combination of IL-2, TNF-α, and IL-6, and after
23 days were double stained with anti-CD45RA-FITC and anti-
15   CD45RO-PE antibodies.

Figure 5. Expression of IFNc and IL-4 mRNA by cytokine-
activated T cells. Purified CD4* CD45RO* resting T cells are
cultured with IL-2 alone for 60 (lane 1) and 100 h (lane 3)
20   or with IL-2, TNF-α, and IL-6 for 60 (lane 2) and 100 h
(lane 4) as described in Materials and Methods. (Lane 5)
Positive template; (lane 6) negative control.

Figure 6. Frequency of resting T cells that grow in
25   response to cytokine combination. CD45RO* resting T cells
were plated in the presence of purified autologous
macrophages, anti-DR mAb with IL-2 alone (*closed circles*) or
in combination (*open circles*) with TNF-α and IL-6. (*Dotted
lines*) 95% confidence limits.

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Detailed Description of Embodiment

Materials and Methods

35   *Purification of Resting T C lls.*   After Ficoll-Hypaque
(Pharmacia) s parati n of PBMC from buffy coats f healthy
donors, most macr phages were removed by plastic adherence.
To obtain a pure resting CD4* T cell population, cells were

8

incubated with a cocktail of mAbs against HLA-DR (L-243; American Type Culture Collection [ATCC], ~~Rockville, MD~~), CD19 (4GT), CD16 (B73.1), CD56 (MY31), CD57 (HNK-1, ATCC), CD8 (OKT8, ATCC), CD11b (OKM-1, ATCC), CD14 (MØ-P9), TCR-c/δ
5 (B1, a gift of G. De Libero, ZLF Basel, Switzerland), CD25 (2A3), CD69 (L78), and CD71 (L01.1). After 30-min incubation on ice, cells were washed twice and incubated with magnetic beads (DYNABEADS ~~Dynabeads~~; Dynal, Oslo, Norway) conjugated with goat anti-mouse IgG and rat anti-mouse IgM,
10 at a 1:4 target/bead ratio. After 30-min incubation, bead-bound cells were removed using rare earth magnet (Advanced Magnetics, Inc., Cambridge, MA). Remaining cells were further purified with four more incubations with beads at increasing target/bead ratios (1:10 to 1:100). Final
15 population was used as a source of resting CD4$^+$ T cells when >99.3% of the population was TCRα/β$^+$ (WT/31) and CD4$^+$ (Leu 3a), as determined by immunofluorescence analyses using a FACScan$^®$ flow cytometer (Becton Dickinson & Co., Mountain View, CA), and fulfilled the following criteria; (a) small
20 size at the FACS$^®$ scatter; (b) absence of FACS$^®$-detectable levels of the activation markers (CD69, CD71, MHC-DR and IL-2 receptor p55 chain (CD25); (c) absence of cells in the S and G$_2$/M parts of the cell cycle; and (d) no significant incorporation of [^{3}H]thymidine when exposed to IL-2. In
25 some experiments resting cells were further negatively sorted as CD45RO$^-$ (adding the mAB UCH1-1) or CD45RA$^-$ (adding the mAB L48). If not otherwise indicated, all the mAbs were from Becton Dickinson & Co.

30 *Preparation of Supernatants.* T cells (5 x 10^5/ml) from a tetanus toxoid (TT)-specific clone were cultured with autologous macrophages (2.5 x 10^5/ml) that had been prepulsed with or without TT (3 μg/ml) (Biocine Sclavo, Siena, Italy). After 16 h, supernatants were collected and
35 filtered with 0.2-μm filters. Culture medium has been previously described (3) using 5% human serum r plasma. Effective supernatants were prepared using medium with either 5% human serum (from Florence blood bank) or serum-

9

fr e media (HL-1: Ventrex, P rtland, OR). Similar results
were obtained with resting T cells derived fr m PBMC of six
different healthy individuals and with supernatants from
activated CD4⁺ T cell clones, with different specificity
5   (purified protein derivatives [PPD] or pertussis toxin),
from four different persons (see Fig. 2 and data not shown).

Cell Cycle Analysis. This was performed as described (4)
using propidium iodide in combination with anti-CD4 mAb
10   (FITC labelled) staining. Analyses were performed with the
FACScan⁰ Lysis II software and doublet discrimination
program (Becton Dickinson & Co.).

Purification of B Cells. PBMC-derived B cells were stained
15   with FITC-labelled anti-CD19 mAb and purified by positive
sorting with FACStar⁰ (Becton Dickinson & Co). Purity was
>98% as determined by staining with anti-CD20 and anti-Ig.

Helper Assay. Noncognate helper assays were performed as
20   previously described (5). Briefly, purified autologous
PBMC-derived B cells (2 x 10³/well) were cocultured for 12 d
with CD4⁺CD45RO⁺ resting T cells (3 x 10⁴/well) in the
presence of cytokine combinations as described (see Fig. 3)
or on anti-CD3-coated plates. To avoid an effect of
25   cytokines on B cell differentiation, plates were washed
after 4-d culture and cytokine combinations were replaced
with IL-2 alone. Ig in the supernatants was measured by
ELISA (5).

30   *Activation of Resting T cells by Supernatants.* Resting T
cells were cultured in 96-well flat-bottom plates (5 x
10⁴/well) with supernatant (50% vol/vol) from T cell clones
cultured with autologous macrophages prepulsed with Ag,
medium or rIL-2 (Cetus Corp., Emeryville, CA) at a
35   concentrati n corr sponding to that found in the T cell
supernatants (i.e. 200-300 U/ml). Activation was measured
at various time points as expression of CD69 and CD25 of
[³H]thymidine incorporation. In some experiments,

[³H]thymidine incoporation of resting CD45RO⁺ or CD45RA⁺ T cells was measured in the presence of different concentrations of IL-2 plus either 1 μg/ml LPS (Difco, Detroit, MI) or supernatant (50% vol/vol) from LPS-activated

5   macrophages. For the preparation of activated macrophage supernatant, 5 x 10⁵ macrophages were simulated with 1 μg/ml LPS (for 6-8 h). [³H]Thymidine incorporation experiments were performed as described (5). The results represent the mean of triplicate wells and SD was always 15%.

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Activation of Resting T Cells by Recombinant Cytokines. Resting T cells (5 x 10⁴/well) in 96-well flat-bottom microplates were cultured for 8 d with various combinations of the following rIL-2 (200-300 U/ml), rIL-6 (500 U/ml;

15 Ciba-Geigy, Basel, Switzerland; IL-6 units were determined with the B9 assay), TNF-α (25 ng/ml; Genzyme Corp., Cambridge, MA), and supernatant (50% vol/vol) from LPS-stimulated macrophages. Thymidine incorporation and CD69 expression were measured as described in Fig. 2. IL-1b (up

20 to 100 ng/ml, Biocine Sclavo Siena, Italy) in combination with IL-2 and TNF-α did not have any activities (data not shown). Recombinant cytokines from two different sources have been used with similar results. The optimal concentration of cytokines was established in preliminary

25 dose-response experiments.

PCR-assisted mRNA Amplification. Purified resting CD4⁺ CD45RO⁺ T cells were cultured with TNF-α plus IL-6 plus IL-2, or IL-2 alone. Total RNA was isolated after 60-100 h of

30 culture from 5 x 10⁵ cells, by RNAzol* B (Biotecx Laboratories, Houston, TX). cDNA was synthesized with murine reverse transcriptase as described (5). ß-actin, IL-4, and IFN-c specific primer pairs were purchased from Clontech (Palo Alto, CA). PCR was performed as described

35 (5).

Limiting Dilution Analyses. CD45RO⁺ resting T cells were plated at different numbers in T rasaki plates (64 wells per

11

condition) in 20μl vol in the presence of purified
autologous irradiated (2,500 rad) macrophages (3 x
10³/well)m anti-DR mAb (L243, 20 μg/ml) with IL-2 alone (300
U/ml) or in combinations with TNF-α (25 ng/ml) and IL-6 (500
5   U/ml). On day 14, cultures were visually inspected for
growth. Randomly selected growing wells were positively
stained with anti-CD4 and anti-TCR-α/β antibodies.
Frequency analyses were done by the least squared method
(6).

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Results and Discussion

A critical point of this study was to use a resting
population devoid of activated T cells that would respond to
15 IL-2 alone. We chose to work with resting CD4⁺ T cells
because, at variant with some CD8⁺ or c/δ T cells with
resting phenotype, they do not express IL-2 receptor p75-
chain in the absence of the p55-chain (7), which may be
responsible for unwanted proliferation responses to IL-2 (8)
20 and for which we did not have a good antibody to sort out.
We therefore performed multistep exhaustive purifications to
obtain highly purified resting CD4⁺ T cells from PBMC (Fig.
1). In preliminary experiments, resting CD4⁺ T cells were
cultured with supernatants from CD4⁺ T cell clones that had
25 been activated with Ag-pulsed macrophages. A representative
experiment in Fig. 2 shows that a fraction of resting CD4⁺
T cells is activated by the supernatant, but not by IL-2, to
express CD69 (9) (Fig. 2A) and IL-2 receptor p55-chain (Fig.
2B), and to incorporate [³H]thymidine (Fig. 2C).

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Since the activating supernatant is produced by the
coculture of two cell types, we sought to determine the
relative contribution of soluble factors produced by T cells
and APCs. For this experiment, resting CD4⁺ T cells were
35 further purified as CD45RO⁺ (memory) and CD45RA⁺ (naive)
subpopulati n (10), since they may have different activation
requirements as already reported for TCR-mediated activation
(11, 12). Fig. 2D shows that supernatant from LPS-activated

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macrophages alone, as IL-2 alone, did not have any activity,
whereas macrophage supernatant in combination with IL-2
induced thymidine incorporation in both CD45RA* and CD45RO*
resting T cells. These results demonstrate that IL-2 and
5   soluble factor(s) produced by APCs are required for the
activation of resting T cells.

To identify the APC-derived factor(s), we tested the effect
of recombinant cytokines known to be produced by macrophages
10 and to have costimulatory activity on T cells, i.e., IL-1ß,
IL-6 and TNF-α (13-15). In the absence of IL-2 all the
possible combinations of these cytokines did not show any
activity over a wide range of concentrations (data not
shown). Fig. 3A shows that TNF-α in combination with IL-2
15 induced resting CD45RO* T cells to express CD69 and to
incorporate thymidine, whereas IL-6 in combination with IL-
2 was much less effective. Remarkably, TNF-α and IL-6, in
combination with IL-2, had a synergistic effect leading to
a stronger activation. A similar effect of IL-2, IL-6, and
20 TNFα was also observed on CD45RA* resting T cells (Fig. 3B),
although, in this case, all three cytokines were required to
induce activation. Furthermore, the cell cycle analyses in
Fig. 3C show that at day 7 of culture   8% of both CD45RO*
and CD45RA* T cells are in the S or G_2/M phases of the cell
25 cycle. Activation of cytokines, measured as expression of
activation markers, thymidine incorporation, or entry into
cell cycle, was never inhibited by mAbs specific for DR,
CD4, or CD3 (data not shown), thus confirming that TCR
signalling is not involved in this type of activation.

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It is interesting to note that we have observed that CD45RA*
T cells activated by cytokines do not switch their phenotype
to CD45RO, as was reported to occur within a few days after
TCR engagement (16). CD45RA* T cells activated by
35 combination of IL-2, TNF-α, and IL-6 were double stained
with anti-CD45RA and anti-CD45RO antibodies at 3-d intervals
up to day 23 of culture. We never found single positive
CD45RO* cells at any time point, and only found a few

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percent f double positive CD45RA⁺ ᵇⁱᵍʰ/CD45RO⁺ ᵈᵘˡˡ. Indeed,
Fig. 4 shows that naive T cells even 23 d after cytokine
activation, when most cells are blastic and express CD69
(data not shown), are mainly CD45RA⁺. The same cells
5 activated with anti-CD3 switched in few days to the CD45RO⁺
CD45RA⁻ phenotype (data not shown).

We next asked whether resting T lymphocytes can be activated
by cytokines to display effector function. We performed
10 PCR-assisted mRNA amplification for lymphokines. Fig. 5
shows that both IFN-c and IL-4 mRNA are expressed by CD45RO⁺
T cells cultured with IL-2, TNF-α, and IL-6, but not with
IL-2 alone. Moreover, CD45RO⁺ T cells activated by cytokine
combination are as effective as anti-CD3-stimulated T cells
15 in helping B cells to produce Ig (Table 1).

TABLE 1.   Resting CD45RO⁺ T Cells Activated by Cytokines
Can Provide Help to B Cells

	IgM	IgG	IgA
		ng/ml	
B cells cocultured with:			
IL-2 plus TNF-α plus IL-6	<15	<5	<10
T cells plus medium	<15	<5	<10
T cells plus IL-2	<15	<5	<10
T cells plus IL-2 plus TNF-α	32	23	<10
T cells plus IL-2 plus IL-6	<15	31	28
T cells plus IL-2 plus TNF-α plus IL-6	75	274	308
T cells plus anti-CD3 mAb plus IL-2	235	219	413

To exclude the possibility that T cell help to B cells could
be due to activation of autoreactive cells, at the end of

14

the helper assay, the B cells were remov d by sorting, and
the CD4* T cells were tested in proliferation against
autologous purified B cells or macrophages. We never found
any autoreactive proliferation (data not shown).

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Neither cytokines nor anti-CD3 induced CD45RA* T cells to
produce IFN-c (<1 IU/ml) and to help B cells (data not
shown). Thus, we conclude that, similar to TCR-mediated
activation (17), cytokines recruit CD45RA* T cells to
10 proliferate but not to help Ig production, whereas they
activate resting CD45RO* T cells to proliferate and display
effector functions.

To evaluate the frequency of resting T cells with memory
15 phenotype that could be stimulated by cytokines to grow, we
performed limiting dilution experiments. CD45RO* CD4*
resting T cells were cultured with IL-2 alone or in
combination with TNF-α and IL-6, in the presence of
autologous irradiated macrophages and anti-DR antibodies to
20 prevent autoreactive responses. Fig. 6 shows that 1 of 33
resting CD45RO* CD4* T cells grew to a visible clone in
response to IL-2, TNF-α. and IL-6. At present we do not
know why only 3% of cells grew in response to cytokines.
The cells that proliferated could have been a subset of
25 resting T cells or could have been at a different stage of
maturation/activation. It is possible that many cells
(≈20%) respond to cytokines and express activation markers.
Some of these cells will display effector functions and only
a minority (3%) will be able to grow in vitro to a clone of
30 visible size.

TNF-α and IL-6 both have been shown to upregulate IL-2R
expression on T cells (15, 18). This could be a possible
mechanism for the activation of resting T cells by this
35 cytokine combination. However, resting T cells cultured for
1-3 d with TNF-α and IL-6, and washed and cultured for 4-5
d more with IL-2, did not show FACS^e-detectable levels of
IL-2R (p55) (data not shown), whereas IL-2R was expressed

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on ≈20% of the same cells cultured with TNF-α, IL-6, and IL-
2 from the beginning of the culture. This experiment,
however, does not rule out the possibility that low levels
of IL-2R below the FACS® sensitivity, are expressed and
5 functionally relevant. Indeed, it has been reported that
Il-2 is required for induction of IL-2R by TNF-α or IL-6
(19). Furthermore, IL-2 augments not only expression of its
own receptor (20) but also upregulates TNF-α receptor (21).
Elucidation of the mechanism of activation of resting T
10 cells by cytokines will require additional biochemical and
molecular analyses.

This novel Ag-independent pathway of T cell activation may
play two important roles in vivo, by recruiting effector T
15 cells at the site of immune response and by maintaining the
peripheral pool of memory T cells. A scenario could be
depicted where resting T cells at sites of Ag-specific
response are activated by cytokines produced by specific T
cells and macrophages to proliferate and to secrete other
20 lymphokines that can further amplify the response. Indeed,
the frequency of resting CD45RO⁺ T cells that respond to
cytokine combination is definitely higher than the usual
frequency of T cells primed by any known Ags.

25 It has been postulated that memory can be carried by long-
lived clones consisting of short-lived cells that require
repeated, intermittent stimulation by persisting Ag, by
recurrent infection, or by cross-reacting environmental Ags
(22-24). In the light of our results, it is tempting to
30 speculate that memory T cells may not require antigenic
stimuli to maintain their clonal size, since resting T cells
with memory phenotype (CD45RO⁺) can be expanded by cytokines
secreted during responses to unrelated antigens. On the
other hand, cytokines can induce proliferation of naive
35 cells without switch to memory phenotype and may therefore
help to maintain the naiv (CD45RA⁺) T cell repertoire.

It will be understood that the invention is described ab ve

by way of exampl only and modifications within the sc pe
and spirit of the invention may be made.

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